

## cDNA Cloning of the B Cell Membrane Protein CD22: A Mediator of B-B Cell Interactions

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### Summary

We have cloned a full-length cDNA for the B cell membrane protein CD22, which is referred to as B lymphocyte cell adhesion molecule (BL-CAM). Using subtractive hybridization techniques, several B lymphocyte-specific cDNAs were isolated. Northern blot analysis with one of the clones, clone 66, revealed expression in normal activated B cells and a variety of B cell lines, but not in normal activated T cells, T cell lines, HeLa cells, or several tissues, including brain and placenta. One major transcript of ~3.3 kb was found in B cells although several smaller transcripts were also present in low amounts (~2.6, 2.3, and 1.6 kb). Sequence analysis of a full-length cDNA clone revealed an open reading frame of 2,541 bases coding for a predicted protein of 847 amino acids with a molecular mass of 95 kD. The BL-CAM cDNA is nearly identical to a recently isolated cDNA clone for CD22, with the exception of an additional 531 bases in the coding region of BL-CAM. BL-CAM has a predicted transmembrane spanning region and a 140-amino acid intracytoplasmic domain. Search of the National Biological Research Foundation protein database revealed that this protein is a member of the immunoglobulin super family and that it had significant homology with three homotypic cell adhesion proteins: carcinoembryonic antigen (29% identity over 460 amino acids), myelin-associated glycoprotein (27% identity over 425 amino acids), and neural cell adhesion molecule (21.5% over 274 amino acids). Northern blot analysis revealed low-level BL-CAM mRNA expression in unactivated tonsillar B cells, which was rapidly increased after B cell activation with *Staphylococcus aureus* Cowan strain 1 and phorbol myristate acetate, but not by various cytokines, including interleukin 4 (IL-4), IL-6, and  $\gamma$  interferon. In situ hybridization with an antisense BL-CAM RNA probe revealed expression in B cell-rich areas in tonsil and lymph node, although the most striking hybridization was in the germinal centers. COS cells transfected with a BL-CAM expression vector were immunofluorescently stained positively with two different CD22 antibodies, each of which recognizes a different epitope. Additionally, both normal tonsil B cells and a B cell line were found to adhere to COS transfected with BL-CAM in the sense but not the antisense direction. Based on the similarity of BL-CAM to other homotypic cell adhesion molecules together with our transfection data, we conclude that BL-CAM may be an important mediator of B-B cell interactions and may play a role in the localization of B lymphocytes in lymphoid tissues.

Subtractive cDNA cloning has been successfully used to isolate genes that are expressed in a tissue-specific manner (1-3). The success of this technique depends upon using cells or tissues whose mRNA populations are very similar. B and T lymphocytes differ in only a small fraction of their expressed genes. For example, B and T cell tumors have been estimated to express only 200-300 unique genes (present in one cell type and not in another (4)). We have been interested in characterizing some of the genes that are expressed uniquely in human B lymphocytes and have used subtractive cDNA cloning to isolate a series of cDNA clones that, on the basis of their mRNA expression, are present in B lymphocytes and not in a wide variety of other cell types. We have reasoned

that these B cell-specific genes will encode for proteins important in human B cell function and, in addition, they will provide useful tools for understanding the basis of their tissue-specific expression.

One of these cDNA clones encodes for a protein that is homologous with several homotypic cell adhesion molecules, appears to mediate cell adhesion among human B cells, and reacts with antibodies to the CD22 membrane antigen. We have referred to this protein as B lymphocyte cell adhesion molecule (BL-CAM).<sup>1</sup> While our work was in progress an-

<sup>1</sup> Abbreviations used in this paper: BL-CAM, B lymphocyte cell adhesion molecule; CEA, carcinoembryonic-associated antigen; MAG, myelin-associated glycoprotein; SAC, *Staphylococcus aureus* Cowan strain 1.

other group isolated a cDNA for CD22 by expression cloning in COS cells using CD22 antibodies. COS cells transfected with CD22 were shown to mediate adhesion to monocytes and mouse erythrocytes (5). The BL-CAM cDNA extends further 5' and 3' than this cDNA and includes an additional 531 bp in the coding region that encodes for 177 amino acids and two immunoglobulin-like domains. The significance of these findings are discussed.

## Materials and Methods

**Cell Culture and Cell Lines.** Human tonsils were obtained from 5–16-yr-old patients undergoing routine tonsillectomies for chronic tonsillitis. B cell-enriched populations were obtained from tonsillar mononuclear cells by twice rosetting with aminoethylisothiuronium bromide-treated SRBCs to remove T cells as previously described (6). The B cell preparations were routinely >96% positive for CD20, <1% CD3<sup>+</sup>, and <1% esterase positive. B cells were stimulated *in vitro* by the addition of 0.005% *Staphylococcus aureus* Cowan strain I (SAC) to the culture media (RPMI 1640 plus 10% FCS). The SAC was obtained from Gibco-Bethesda Research Laboratories (Gaithersburg, MD) and washed four times with PBS before use. The cell lines HS-Sultan, Ramos, IM-9, Jurkat, and Hut 102 were obtained from the American Type Culture Collection (Rockville, MD). The Epstein Barr virus-transformed cell line EBV-1 was derived from a spontaneous transformation of tonsil B cells. The CEM, Sup T1, MJ, and MT-4 T cell lines were a kind gift of Dr. Scott Koenig (NIAID, NIH, Bethesda, MD), the BJA-B cell line was a gift from Dr. Edward Oates (University of Miami, Miami, FL), and the NALM-6 pre-B cell line was a gift from Dr. Thomas Tedder (Harvard University, Boston, MA).

**Synthesis of Hybrid-subtracted cDNA Probe.** Poly(A) mRNA from PHA (2 µg/ml) and PMA (10 ng/ml)-activated peripheral blood T cells (36 h) and from SAC and PMA (10 ng/ml)-activated tonsil B cells (36 h) were prepared from guanidine thiocyanate-purified RNA using oligo(dT) columns (Collaborative Research, Lexington, MA). The subtracted probes were generated according to established methods (7). Briefly, the first strand B cell cDNA was prepared using 5 µg of poly(A) mRNA in the presence of 1 mCi of <sup>32</sup>P-dCTP. The RNA was hydrolyzed and the cDNA purified over a G50 column. tRNA (20 µg) and poly(A) T cell mRNA (10 µg) were added to the labeled cDNA. The cDNA and mRNA were ethanol precipitated and resuspended in 4 µl of water and 4 µl of phosphate buffer (1 M phosphate, pH 6.8; 0.2% SDS; and 10 mM EDTA) in a silanized eppendorf tube. The hybridization mixture was overlaid with 100 µl of paraffin oil, heated to 100°C for 1 min, and incubated overnight at 68°C. The single-stranded DNA and double-stranded DNA-RNA complex were separated on a hydroxylapatite column (Bio-Rad Laboratories, Cambridge, MA) and subsequently, the single-stranded DNA was purified on a G50 column. The procedure was repeated a second time to increase the efficiency of the subtraction. Typically, the first hybridization resulted in a 80–90% subtraction and the second hybridization resulted in a 30–50% subtraction. The overall subtraction was 90–96%.

**Synthesis of cDNA Library and Plaque Screening.** A combined oligo(dT) and random primed cDNA library in λ zap (~3 × 10<sup>6</sup> primary clones before amplification) was generated with the use of poly(A)-containing mRNA from normal tonsillar B lymphocytes stimulated for 36 h with SAC and PMA (8; Stratagene, La Jolla, CA). The library was plated at low density (4,000 plaque-forming units/150-mm plate), and the nitrocellulose membrane lifts were hybridized for 36 h at 68°C with subtracted cDNA probes (10<sup>6</sup> cpm/ml) in hybridization solution (5× SSPE, 5× Denhardt's,

100 µg/ml of salmon sperm DNA, and 10% dextran sulfate). The filters were washed for 20 min with 2× SSPE 0.1% SDS twice at room temperature and twice at 55°C. Positive plaques were identified and rescreened with a second subtracted probe. The secondary clones were rescreened with a B cell cDNA probe or a T cell cDNA probe. Those clones hybridizing with the B cell probe but not the T cell probe were rescreened with Ig (IgG, IgM, κ, λ, IgA) probes, and positive plaques were eliminated. Approximately 200 clones were isolated that survived this screening. These clones were further characterized by Northern blot analysis and partial DNA sequencing. Clone 66 was one of these clones that was expressed in B cells and not T cells, and was found to be a previously uncharacterized gene. The original clone 66 was used to rescreen the B cell cDNA library and 10 hybridizing clones were isolated. Two clones, 66.2 and 66.5, were each 3.2 kb and were used for further studies.

**DNA Sequencing of BLCAM and Analysis of Predicted Protein.** DNA sequencing was performed on double-stranded DNA templates using the dideoxy chain termination technique with Sequenase following the manufacturers protocols (U.S. Biochemical Corp., Cleveland, OH). Generated sequence information was used to synthesize new primers to complete the sequencing. Oligonucleotides were synthesized using an oligonucleotide synthesizer (Applied Biosystems, Inc., Foster City, CA). Each strand of 66.2 was sequenced. The sequencing project was performed with the aid of the Assemblage program in PCGENE (Intelligenetics, Mountain View, CA). The DNA (Genbank) and protein (National Biomedical Research Foundation [NBRF]) data base searches and protein alignments were performed with the FASTA, FASTP, and Align programs, respectively (9, 10), at the Advanced Scientific Computing Laboratory (Frederick, MD). The BL-CAM-predicted sequence was analyzed with the aid of the Prosite, Signal, and SOAP programs of PCGENE (Intelligenetics).

**In Vitro Transcription and Translation.** Capped RNA was synthesized in a 25-µl reaction using as a substrate 2 µg of linearized DNA for 1 h at 40°C using T3 or T7 RNA polymerase (Stratagene) in the presence of RNasin. The capped mRNA transcripts were visualized on a formaldehyde/agarose gel and found to be the appropriate size. *In vitro* translation of the RNAs was done using a rabbit reticulocyte lysate (Stratagene) in a 25-µl reaction using 10% of the capped mRNA in the presence of <sup>35</sup>S-methionine. The translation products were separated on a 8% SDS-PAGE gel and visualized by autoradiography.

**Plasmid Construction and Transient Expression in COS-7 Cells.** Recombinant 66.2 was subcloned into CDM8 in the sense and antisense orientations. A 2.8-kb XhoI/SSPI insert of 66.2 was blunted with Klenow fragment, linked with BSTX1 linkers (Invitrogen, San Diego, CA), and ligated into the BSTX-1 site in CDM8. The ligation mix was used to transform P3 cells. The termination sites of the sense and antisense constructs were sequenced using the dideoxy chain termination technique, which confirmed the orientations. The recombinant plasmids were purified on Qiagen columns (Qiagen Inc., Studio City, CA) and used to transfect COS-7 cells via electroporation (11). 5 × 10<sup>6</sup> COS cells from a subconfluent flask were washed with electroporation buffer (272 mM sucrose, 7 mM sodium phosphate, pH 7.4, and 1 mM MgCl<sub>2</sub>) and resuspended at 7 × 10<sup>6</sup> cells/ml in the same buffer. The cells were transferred to a sterile 0.4-cm electroporation cuvette (Bio-Rad Laboratories), and 100 µl of a 100-µg/ml DNA suspension in electroporation buffer was added. The cuvette was incubated on ice for 10 min and then electroporated at 350 V and 25 microfarads (µFD) with a Gene Pulser (Bio-Rad Laboratories). After pulsing, the cells were returned to ice for 10 min. The cells were resuspended

in DMEM/10% FCS and cultured at 37°C overnight. The following day, the cells were harvested with trypsin/EDTA and then replated in six-well tissue culture plates or slide chambers (Lab-Tek Products, Naperville, IL). 48 h later, the cells were used for FACS (FACS is a registered trademark of Becton Dickinson & Co.) analysis or functional studies. For FACS analysis, the cells were harvested with versene and immunofluorescently stained with CD22 (HD6 and HD39; Boehringer Mannheim Biochemicals, Indianapolis, IN) and a FITC-conjugated goat anti-mouse IgG (Tago Inc., Burlingame, CA), and analyzed on a EPICS analyzer (Coulter Electronics, Inc., Hialeah, FL). For functional studies, the transfected COS cell monolayer was washed with DMEM without serum; the test cells, in DMEM, were added in the presence or absence of antibody; the cells were allowed to adhere for 1–2 h; the monolayer was gently washed eight times with DMEM; and the monolayer was examined with an inverted microscope. In some cases, the transfected COS cell monolayer was directly stained with CD22 or a control antibody followed by the developing antibody and then visualized with an inverted fluorescent microscope.

**Northern Blots and In Situ Hybridization.** For northern blots, total RNA was prepared by a guanidine thiocyanate method (12). The RNA was size fractionated on 1% agarose/formaldehyde gel, transferred to nitrocellulose, UV crosslinked, and hybridized in a formamide-based hybridization solution at 42°C with random primed, <sup>32</sup>P-labeled cDNA inserts. The filters were washed twice at room temperature in 2× SSC/0.1% SDS for 20 min and twice at 55°C in 0.1× SSC/0.1% SDS, and exposed to film (12).

For in situ hybridization, a protocol was used similar to that described by Pardue (13) for RNA hybridization. Paraffin-fixed tonsil sections were mounted on silanized slides after which the slides were deparaffinized, hydrated, and washed for 20 min in 0.1 M HCl and followed by digestion with proteinase K (10 µg/ml) for 30 min. The slides were acetylated by treatment with acetic anhydride in a 0.1 M triethanolamine buffer, pH 8.2, and prehybridized at 45°C for 2 h in a solution of 50% formamide, 10% Denhardt's solution, 0.5 mM EDTA, 10 mM Tris-HCl, 5 mM DTT, 0.3 M NaCl, and 50 µg/ml of yeast tRNA. Hybridization was performed in the same solution with the addition of an equal volume of 20% dextran sulfate in 50% formamide and probe at  $8 \times 10^7$  cpm/ml. 1 µl of hybridization solution was used per 25 mm<sup>2</sup> of specimen. Ordinary coverslips were sealed at the edges and the slides hybridized at 42°C. <sup>35</sup>S-labeled sense and antisense RNA probes were made from the bluescript BL-CAM plasmid using the T3 (sense) and T7 (antisense) promoters by Lofstrand Laboratories (Gaithersburg, MD). The probes were hydrolyzed in carbonate buffer to a length of ~300 bp. They were purified by repeated precipitation with ethanol and had specific activities of  $\sim 2 \times 10^6$  cpm/µg. After overnight hybridization, the coverslips were removed and the slides were washed once in 50% formamide 1× SSC with 1 mM EDTA and 5 mM DTT followed by five washes at 60°C with 2× SSC with EDTA and DTT followed by digestion of single-strand RNA with RNase A and RNase T1. Slides were then washed in 0.3 M ammonium acetate in alcohol and dried overnight. Slides were dipped in NTB2 emulsion (Eastman Kodak, Rochester, NY), exposed for 3 d, and developed in Kodak D-19 diluted 1:1. Fixed slides were stained with hematoxylin and eosin and examined at 100× with darkfield illumination on an adapted microscope (Carl Zeiss, Inc., Thornwood, NY).

## Results

**Isolation of Clone 66 and DNA Sequence Analysis.** A B lymphocyte cDNA library was constructed from mRNA purified

from human B lymphocytes stimulated with SAC and PMA. Approximately 20,000 clones were screened with subtractive cDNA probes generated from human B and T cell mRNA. One of the isolated clones was found to hybridize to a 3.3-kb mRNA present in total RNA isolated from B cells but not from T cells. Partial DNA sequence analysis and search of Genbank with the derived sequence information did not reveal any significant homologies to known DNA sequences. This clone was used to rescreen the cDNA library and 10 clones were isolated, two of which appeared full length. One of the clones, 66.2, was fully sequenced in both directions and the derived DNA sequence information is presented in Fig. 1. Of note is a poly CA tract present in the 3' untranslated portion of the cDNA. Similar poly CA tracts have been found in a variety of other mRNAs, including the CEA mRNA, and it is thought to function as a weak enhancer element (14, 15). The clone 66.2 DNA sequence, although extending further 5' and 3', is essentially identical to a cDNA for CD22 isolated by Stamenkovic and Seed (5), with the exception that their CD22 cDNA clone lacks 531 bp present in clone 66.2. The two sequences diverge at bp 777 and reassume identity at bp 1308 in the BL-CA MDNA sequence. Analysis of the BL-CAM cDNA revealed an ATG-initiated open reading frame of 2,541 bases, which encodes for a predicted protein of 847 amino acids with a molecular mass of 95 kD. In contrast, the published CD22 cDNA clone is predicted to encode for a 67-kD protein (5). In vitro transcription and translation of the BL-CAM cDNA demonstrated a protein that migrated at a molecular mass of 95 kD on SDS-PAGE (Fig. 2). The probable initiation ATG was followed by a signal peptide predicted to be either 21 or 22 amino acids. Hydrophobicity plots revealed the presence of the signal peptide and a single membrane-spanning domain (amino acids 688–706) with a predicted intracytoplasmic region of 140 amino acids. Because of an additional G residue at bp 2541 of the clone 66.2 cDNA, the predicted intracytoplasmic portion of BL-CAM is 23 amino acids longer than predicted by the Stamenkovic and Seed cDNA (5). The intracytoplasmic region of BL-CAM has a predicted protein kinase C phosphorylation site (threonine 764), a casein kinase II phosphorylation site (threonine 789), and six tyrosines. None of the intracytoplasmic tyrosines matches the consensus sequence (R,K-x2-[E,D]-x3-Y) for a tyrosine phosphorylation site, although one of the sites, tyrosine 796, is a close approximation to the consensus with an arginine eight amino acids instead of seven to the NH<sub>2</sub>-terminal side of the tyrosine. The predicted extracytoplasmic portion of the molecule has 12 n-linked glycosylation sites, suggesting that the protein is heavily glycosylated, and if all the sites are glycosylated, the estimated molecular mass of the BL-CAM protein would be ~140 kD, which is a close approximation of known molecular mass of CD22 (16).

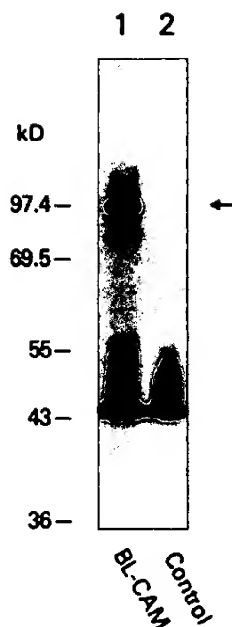
**Expression of BL-CAM mRNA.** To examine the expression of the BL-CAM mRNA in various cell types and to examine the effects of cytokines and B cell activation on BL-CAM expression, we performed a series of Northern blots. A major 3.3-kb mRNA consistent with the size of the BL-CAM cDNA was found to be well expressed in in vitro acti-

1	CCATCCCATG	GTGAGGAGAG	ACAAGGCGAA	ACAGGCTTGC	ACCAGACAC	GACACCAATG	ATCTCTCTGG	CCGCTGGCTG	CTGCTCTGG	90
12	TTCTAGATA	CTTGGCTTTC	TCTGACTCAA	GTAAATGGGT	TTTGGACAC	CCTGAAAGCC	HTLLLG	FWLL	LLLL	180
42	ITPCT	YRA	LDG	DLES	FIL	FHN	FHN	FHN	FHN	270
72	DGT	LYE	STK	DGKV	PSE	QKR	VQFL	GDX	NKN	360
102	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	450
132	INL	NV	STK	STK	STK	STK	STK	STK	STK	540
162	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	630
192	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	720
222	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	810
252	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	900
282	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	990
312	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1080
342	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1170
372	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1260
402	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1350
432	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1440
462	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1530
492	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1620
522	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1710
552	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1800
582	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1890
612	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	1980
642	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2070
672	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2160
702	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2250
732	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2340
762	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2430
792	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2520
822	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2610
	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2700
	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2790
	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2880
	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	2970
	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	3060
	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	3150
	CTLS	INP	VNL	NDSG	QLG	LRM	ESKY	EKW	MER	3240

Figure 1. Sequence of the cDNA representing BL-CAM transcript. Nucleotide sequence of a cDNA containing the entire coding and 3' untranslated region of BL-CAM. The amino acid sequence is numbered on the left and the nucleic acid sequence is numbered on the right. The in-frame termination codons upstream of the predicted initiation site and at the end of the coding sequence are designated by three asterisks. The boundaries of the Ig domains are indicated by numbers that correspond to the number of the domain from the NH<sub>2</sub> terminus. Domains 3 and 4 are underlined. The transmembrane portion of BL-CAM is underlined with a broken line. The polyadenylation signal beginning at adenosine 3221 is also underlined.

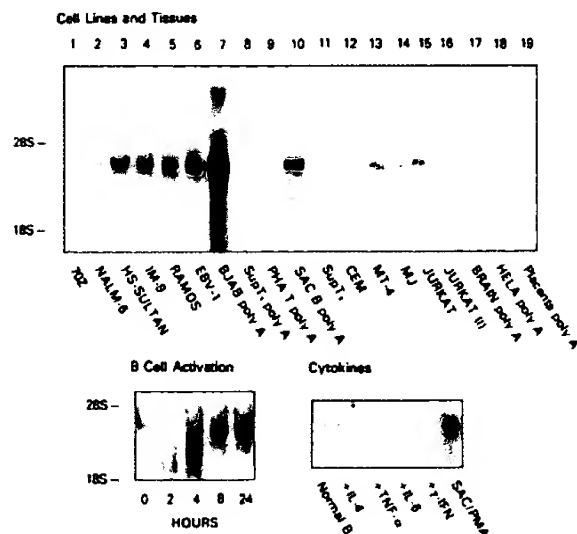
vated normal B cells but to be expressed at low levels in B cells freshly isolated from human tonsils (Fig. 3). No expression was found in tonsillar T cells or tonsillar T cells activated with PHA and PMA. A variety of B cell lines were found to express the BL-CAM mRNA, including the pre-B cell line Nalm-6. Several B cell lymphoma cell lines expressed

the 3.3-kb BL-CAM transcript as well as several minor transcripts. The minor transcripts are likely to be alternatively spliced mRNAs. In contrast, none of the T cell lines, including several human T cell leukemia type 1-transformed lines, were found to express this mRNA. Additionally, no significant hybridization signal was found with poly(A) RNA



**Figure 2.** In vitro transcription and translation of BL-CAM cDNA. The BL-CAM Bluescript plasmid was linearized with XhoI and in vitro transcribed using the T3 (sense) promoter or the T7 (antisense) promoter. The BL-CAM sense or antisense RNA was in vitro translated using a rabbit reticulocyte system and  $^{35}$ S-methionine. The translated products using the sense (lane 1) or the antisense (lane 2) were analyzed on a 10% polyacrylamide and visualized by fluorography.

derived from Hela cells, brain, or placenta (Fig. 3). The effects of several cytokines on normal B lymphocyte expression was also examined. Neither IL-4, IL-6, TNF- $\alpha$ , nor IFN- $\gamma$  enhanced expression of BL-CAM; however, treatment of the



**Figure 3.** The expression of BL-CAM in various tissues, cell lines, and normal B and T lymphocytes. Total RNA was prepared from various tissues, lymphoid cell lines, and normal B and T cells. In some cases, poly(A) RNA was prepared. Each lane contains 15  $\mu$ g of total RNA or 2  $\mu$ g of poly(A) RNA; each of the cell lines is indicated below their respective lane (top). The normal B cells were prepared from tonsils and in vitro activated with SAC and PMA for the indicated time periods (bottom, left) or incubated with 1,000 U/ml of IL-4, 1 ng/ml of TNF- $\alpha$ , 100 U/ml IL-6, 1,000 U/ml of IFN- $\gamma$ , or SAC/PMA for 24 h (bottom, right).

tonsillar B cells with SAC and PMA significantly increased its expression (Fig. 3).

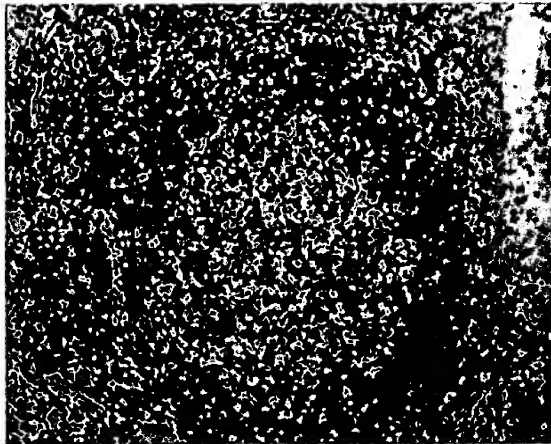
**Homologies to Other Proteins and Domain Structure.** Similar to Stamenkovic and Seed (5), we also noted the similarity of our clone 66.2, BL-CAM, to myelin-associated glycoprotein (MAG). When the BL-CAM-predicted protein was used to search the NBRF protein database using the FASTP search program, it revealed very significant homologies with MAG and carcinoembryonic-associated antigen (CEA). The first 420 amino acids of BL-CAM (two gaps) aligned with the first 414 amino acids of MAG (four gaps) were 27% identical. Amino acids 222–684 of BL-CAM (11 gaps) aligned with amino acids 168–596 of CEA (eight gaps) were 29.5% identical (Table 1). If conservative amino acid substitutions are included in the analysis BL-CAM and MAG are 64% similar, while BL-CAM and CEA are 71% similar over the above regions. Both MAG and CEA are members of the Ig superfamily, and both function as adhesion molecules (17–21). CEA has been modeled and is predicted to contain seven domains with one NH<sub>2</sub>-terminal V-like domain and six C region-like domains, and MAG has been predicted to contain one NH<sub>2</sub>-terminal V-like domain four C region domains (22, 23). Similarly, BL-CAM appears to have a single NH<sub>2</sub>-terminal V-like domain and six C region-like domains. The first two domains each have  $\sim$ 110 amino acids, while the other domains have  $\sim$ 90 amino acids each. The first three domains contain three cysteines, while all the other domains contain two cysteines, except domain 5, which contains four cysteines. There are some similarities between the domains, particularly do-

**Table 1.** Proteins Related to BL-CAM

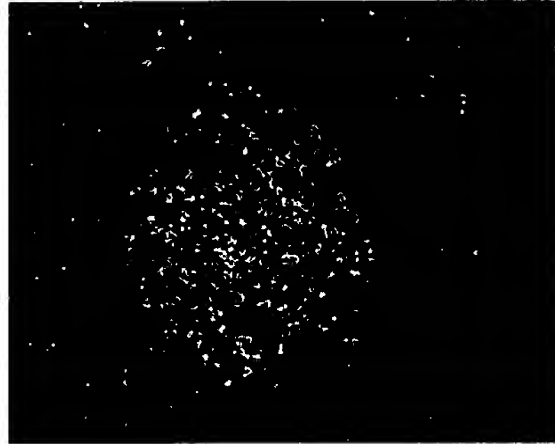
Protein	Optimized alignment score	Amino acid identity
		%
Myelin-associated glycoprotein	361	25.9 over 425 aa
Carcinoembryonic antigen	338	29.5 over 462 aa
Biliary glycoprotein I	221	22.4 over 308 aa
Nonspecific crossreactive antigen	187	32.6 over 138 aa
Myeloid cell surface antigen CD33	184	25.2 over 266 aa
IgG Fc receptor	159	19.6 over 275 aa
Carcinoembryonic antigen-related protein	158	19.9 over 287 aa
Neural cell surface adhesion molecule	150	21.5 over 266 aa

The NBRF protein data base was searched with the BL-CAM amino acid sequence using the FASTP-Lipman-Pearson algorithm, and the eight proteins with the best scores are listed above. Each of the proteins was aligned with BL-CAM using the Align algorithm, and the percent amino acid identity over the aligned portions of the proteins was determined.

A



B



**Figure 4.** In situ hybridization with BL-CAM RNA probe.  $^{35}$ S-methionine-labeled antisense BL-CAM RNA was hybridized to human tonsil tissues and then stained with hematoxylin. *A* was photographed with visible light and *B* was photographed using dark field illumination. When viewed with dark field illumination, a positive signal is light while a negative signal is dark.

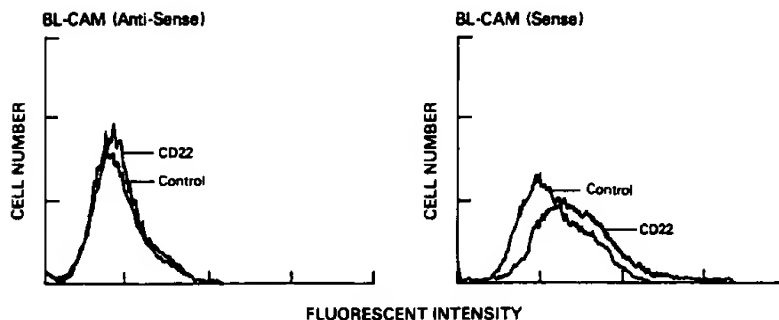
main 3–7. The best match is between domains 3 and 7, which are 33% identical. The CD22 cDNA cloned by Stamenkovic and Seed (5) lacks domain 3 and 4 of BL-CAM.

When the intracytoplasmic portion of BL-CAM was used to search the NBRF protein data base or the translated Genbank data base, no significant homologies with other proteins were found. The multiple phosphorylation sites suggest that it is a phosphoprotein, but whether or not it has a role in intracellular signalling is currently unknown.

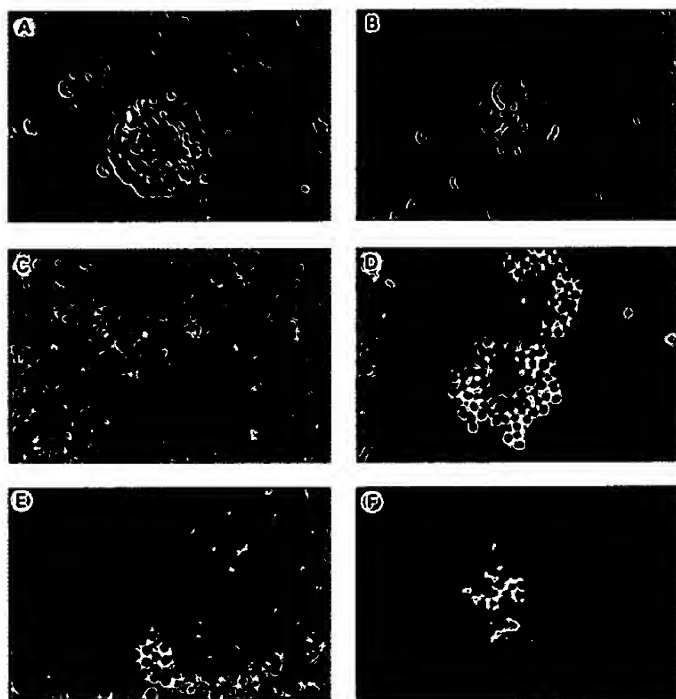
**Analysis of BL-CAM Expression by In Situ Hybridization.** To characterize the expression of BL-CAM mRNA in vivo, we used in situ hybridization to localize its mRNA in a variety of different tissues.  $^{35}$ S-methionine sense and antisense RNA probes were made and sheared to 200–300 bases. In situ hybridization with paraffin-fixed tissues was performed. No significant hybridization signal was found with liver, placenta, kidney, or small intestine, either with the sense or the antisense RNA (data not shown). However, when either tonsil or lymph node tissue was used, a clear hybridization signal

was found with the antisense probe but not with the sense probe. The strongest signal in both the lymph node and tonsil was in germinal centers, and a less intense although clearly present signal was present in the mantle zone; both regions are highly enriched with B lymphocytes (Fig. 4).

**Expression of BL-CAM in COS Cells and Evidence it Functions as a Cell Adhesion Molecule.** Since the BL-CAM protein was predicted to be an integral membrane protein, we determined whether any known mAbs reacted with the expressed protein. The full-length BL-CAM cDNA was subcloned into the CDM8 vector in the sense and the antisense direction. Both constructs were transfected into COS cells via electroporation. 72 h later, the COS cells were immunofluorescently stained with a variety of B cell mAbs, including two CD22 antibodies that recognize different epitopes, and analyzed by fluorescent microscopy and FACS. Examination of the stained COS cells with the fluorescent microscope revealed clear staining of ~5% of the cells with the CD22 antibody (data not shown). FACS analysis also demonstrated CD22 staining



**Figure 5.** Expression of BL-CAM in COS cells and reactivity with CD22 antibodies. COS cells were transiently transfected with sense or antisense BL-CAM CDM8 constructs via electroporation, and 3 d later, immunofluorescently stained with CD22 antibodies and analyzed by a FACS.

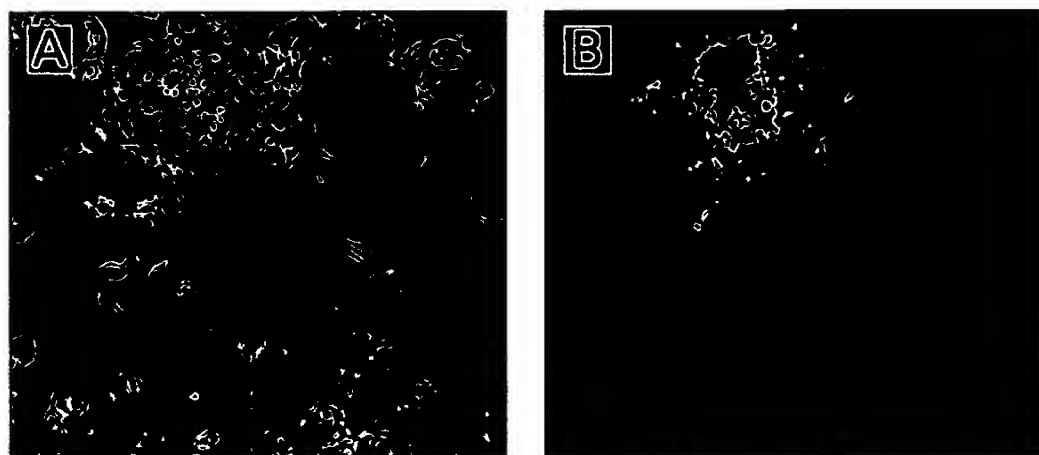


**Figure 6.** Adherence of B cells to COS cells transfected with BL-CAM cDNA. COS cells were transiently transfected with sense or antisense BL-CAM CDM8 constructs via electroporation. 3 d later, the monolayer was overlaid with B cells, washed extensively, examined by inverted microscope for the presence of adherent cells, and photographed. BL-CAM-transfected COS cells were overlaid with HS-Sultan cells (*A* and *B*). Antisense-transfected COS cells were overlaid with tonsil B cells (*C*). BL-CAM-transfected COS cells were overlaid with tonsil B cells (*D* and *E*). BL-CAM-transfected COS cells were overlaid with tonsil B cells previously immunofluorescently stained with CD20 and examined by fluorescent microscopy (*F*).

of the COS cells transfected with BL-CAM in the sense direction but not in the antisense direction (Fig. 5). When the antibodies were used individually rather than together, both antibodies were found to immunofluorescently stain the COS cells transfected with the sense BL-CAM construct (data not shown).

We next tested the hypothesis that BL-CAM mediates B cell-B cell adhesion. COS cells were transfected with the BL-

CAM expression vector in both the sense and the antisense directions. 72 h later, the transfected COS cells were overlaid with HS-Sultan, a B cell line that expressed high levels of CD22, with Jurkat, a T cell line, or with normal tonsillar B lymphocytes. The normal B cells and the HS-Sultan cell line were found to adhere to the COS cells transfected with BL-CAM in the sense orientation (Fig. 6, *A*, *B*, and *D-F*), but not in the antisense orientation (Fig. 6 *C*). The addition



**Figure 7.** Correlation between expression of BL-CAM by transfected COS cells and adhesion of B cells. COS cells were transiently transfected with the sense BL-CAM construct, immunofluorescently stained with a CD22 antibody, overlaid with purified tonsillar B cells, washed, and examined by a inverted microscope for B cell adhesion (*A*) and fluorescence (*B*). *A* was photographed to reveal concurrent expression of BL-CAM by immunofluorescence.

of the B cells to the COS cell monolayer often resulted in the detachment of some of the COS cells, and direct B cell COS cell conjugates were observed (Fig. 6 B). The tonsillar cells adherent to the COS cells were shown to be B lymphocytes by immunofluorescent staining with CD20 before the adhesion step. The cells adherent to the COS cell monolayer were examined with a fluorescent microscope (Fig. 6 F). The Jurkat cells failed to adhere to BL-CAM-transfected COS cells (data not shown). While the difference between the antisense-transfected and BL-CAM-transfected cells was striking, the addition of CD22 antibodies failed to inhibit the binding of the HS-Sultan or tonsil B cells to the transfected COS cells. A variety of other antibodies also failed to inhibit the B cell binding, including LFA-1, CD44, and ICAM-1 (data not shown). The failure of CD22 antibodies to inhibit binding of the B cells to the transfected COS cells allowed us to immunofluorescently stain the COS cell monolayer with CD22 before adherence of the B cells. There was a nearly 1:1 correlation between the COS cells that bound B cells and the expression of CD22 (Fig. 7). Thus, although the transfection data were not conclusive, since the CD22 antibodies failed to block the binding of the B cells, it strongly suggests that one of the functions of BL-CAM, CD22, is to mediate B cell-B cell interactions.

## Discussion

Using subtractive hybridization, we have isolated several cDNAs that identify mRNAs expressed in B cells and not in T cells. One of these clones (clone 66.2) has been fully sequenced and found to encode CD22, a B cell membrane glycoprotein, which is likely to have a role in B cell-B cell interactions and is referred to as BL-CAM. The predicted BL-CAM protein core has a molecular mass of 95 kD, and in vitro transcription and translation of BL-CAM followed by analysis by SDS-PAGE confirmed the predicted molecular mass and agrees with the known molecular mass of deglycosylated CD22 (24, 25). Analysis of the predicted protein revealed some interesting structural features and homologies with previously characterized proteins, and in addition, gave some insights into the probable function of this protein. Functional studies in COS cells transfected with BL-CAM suggest that it functions as an adhesion protein mediating B cell-B cell interactions. These data support and extend the observations made by Stamenkovic and Seed (5).

DNA sequence analysis of clone 66 revealed a 3,260-bp insert and a predicted open reading frame of 847 amino acids. Salient features of the predicted BL-CAM protein included a signal peptide of 21 amino acids, a transmembrane domain of 19 amino acids, and an intracytoplasmic portion of 140 amino acids. The extracytoplasmic portion can be divided into 7 Ig region-like domains of 90–110 amino acids each. The first three domains have an unpaired cysteine residue that could form an intra-chain disulfide bond. Each of the remaining domains contain two cysteine residues, except for domain 5, which has four cysteine residues. The extracytoplasmic portion of BL-CAM is related to several other Ig-like proteins;

the most striking homologies on search of protein data bases were the similarities with CEA and MAG. Interestingly, the first 425 amino acids of MAG aligns with 26% identity with domains 1–4 of BL-CAM, while CEA aligns with 29% identity with domains 3–7 of BL-CAM, thus encompassing the entire extracytoplasmic portion of BL-CAM. When conservative amino acid changes are included in the analysis, MAG and BL-CAM are 64% and CEA and BL-CAM are 71% homologous over these regions. Several other proteins including bilary glycoprotein 1, pregnancy-associated glycoprotein, and nonspecific crossreactive antigen, are members of the Ig super family and are closely related to CEA (50–90% identical at the amino acid level), suggesting that these proteins form a family within the Ig super family (26). While MAG and BL-CAM are more diverged from CEA than these other proteins, their similarity to CEA suggests they may have arisen from a common precursor.

Search of protein databases with the amino acid sequences of the predicted intracytoplasmic domain of BL-CAM failed to identify any relationship to previously characterized intracytoplasmic region of known proteins. However, it does have predicted protein kinase C and casein kinase II phosphorylation sites. In addition, there are six intracytoplasmic tyrosines, one of which is a close match with the consensus for a tyrosine phosphorylation site. These findings suggest some role for the intracytoplasmic region of BL-CAM in signaling. To date, studies with CD22 antibodies have suggested that CD22 may be involved in B cell activation. CD22 antibodies augment entry of B cells into the cell cycle and further increase anti-Ig-induced rises in intrathymic  $Ca^{2+}$  levels (27, 28). Additionally, preliminary studies indicate B cell activation is associated with changes in the phosphorylation status of CD22 (22).

Previous studies with the CD22 antibody have shown that it is expressed on 50–75% of EBV-transformed B cell lines, 50% of B cell lymphomas, and nearly 100% of hairy cell leukemia cells (16). Additionally, B cell differentiation is associated with the loss of CD22 (16). The reasons for this variable expression are unclear, but it will be of interest to characterize the mRNA transcript present in these various cell lines and primary tumors. BL-CAM mRNA expression was examined in five EBV-transformed B cell lines and was present in three cell lines that were CD22 positive, and negative in two cells that were CD22 negative. The predominant BL-CAM mRNA in the positive cells was the 3.3-kb transcript. Interestingly, the expression of BL-CAM correlated with the expression of LFA-1. The two cell lines which were BL-CAM negative also expressed low levels of LFA-1, suggesting that these two genes may be coordinately regulated. These BL-CAM-negative, low LFA-1-expressing cells were strongly PNA positive, suggesting that neither LFA-1 nor BL-CAM accounts for the peanut agglutinin positivity of germinal center B cells (29). Of note, these cells grew in culture as single cells rather than in the large clumps typically seen with EBV-transformed cell lines (J. Kehrl, unpublished observations). We have isolated genomic clones for BL-CAM in order to characterize the promoter region that should help to address the nature of these differences in expression. The



EBV-transformed B cell lines that do or do not express BL-CAM mRNA will be particularly useful in characterizing the promoter region of this gene.

Several lines of evidence support an important role for BL-CAM in interactions between human B cells. BL-CAM is a member of the Ig super family and is highly related to CEA and MAB, two proteins known to mediate homotypic cell adhesion (19-21), suggesting a similar role for BL-CAM in B cells. The expression of CD22 *in vivo* is also consistent with a role for CD22 in maintenance of the cellular architecture in B cell-rich regions. The levels of CD22 are relatively low in peripheral blood B cells and increased in tissue-based B cells (e.g., in the mantle region surrounding germinal center in tonsils and lymph nodes). The quantities of cell adhesion molecules on the cell membrane are an important parameter in their functional capacity to mediate adhesion. Small changes in surface density can lead to large changes in binding rates (30). Immunocytochemistry with CD22 antibodies has also demonstrated considerable expression in germinal centers, although less striking than in the mantle region (16). More direct evidence for BL-CAM as a B cell adhesion molecule was obtained by transfection of a BL-CAM expression plasmid into COS cells. Strongly CD22-positive B cells adhered to the transfected COS cells but not to COS cells transfected with a plasmid in which BL-CAM was oriented in the opposite direction. However, the binding was not inhibited by two CD22 antibodies that recognize two different epitopes. The failure of these antibodies to inhibit binding is likely due to the recognition of an epitope that is not involved in binding. Although our data suggest that BL-CAM is a homotypic cell adhesion molecule (i.e., BL-CAM is both a ligand and a receptor), it is not conclusive and awaits further studies. Preliminary studies with a B cell line transfected with BL-CAM suggests that BL-CAM may mediate B cell adhesion via direct interaction with itself (J. Kehrl, unpublished observation). Additionally, based on the adhesion assays performed by Stamenkovic and Seed (5), BL-CAM in the absence of domains 3 and 4 can interact with other cell surface molecules that are present on monocytes and mouse erythrocytes. The failure of Stamenkovic and Seed (5) to observe B cell adhesion with peripheral blood B cells may be related to a different conformation of the CD22 molecule due to the lack of domains 3 and 4. Alternatively, if CD22 is a homotypic cell adhesion molecule and interacts with other CD22 molecules on opposing cells, the relatively low levels of CD22

on peripheral blood B cells may account for the failure to observe B cell adhesion.

Besides BL-CAM, both LFA-1 and ICAM-1 are important in B cell adhesion as well and can mediate B cell-B cell interactions (31, 32). However, these molecules are expressed on a wide variety of cells, distinct from the B cell-specific expression of BL-CAM, and are less likely to be directly involved in the maintenance of B cell-enriched regions. The role of LFA-1 is more likely to be important in the interaction of B cells with other cells, including monocyte/macrophages and T cells (33). Although the possibility remains that BL-CAM may be a ligand for LFA-1, we have no evidence to support that possibility. LFA-1 antibodies did not block the adhesion of B cells to COS cells transfected with CD22.

The high levels of BL-CAM mRNA in germinal centers compared with the mantle region are somewhat perplexing. *In situ* staining of human tonsil with CD22 antibody reveals a reversed pattern with high levels of expression in the mantle region and less striking levels in germinal centers (16). We have confirmed our results with several different donors in both tonsil and lymph node. Additionally, sense RNA probes did not hybridize in either region. Thus, BL-CAM mRNA expression appears to differ from protein expression as delineated by CD22 mAb staining. A number of potential explanations exist for those observations, including a decreased affinity of CD22 in the germinal centers due to alternative glycosylation or alternative processing of BL-CAM mRNA in the germinal center cells, which alters the binding sites for CD22. Additionally, there is evidence that certain CD22 antibodies recognize dendritic cells in a variety of tissues (34). This raises the possibility that BL-CAM mRNA may be present in the follicular dendritic cells present in germinal centers. Further studies are needed in order to clarify whether BL-CAM is expressed in other cells besides B cells in germinal centers.

In conclusion, we have isolated a full-length cDNA clone that encodes the CD22 protein, BL-CAM, by the use of subtractive hybridization and subsequent expression in COS cells. Functional studies using the COS cell transfectant suggest that BL-CAM is a B cell adhesion molecule and that it may play a role in maintenance of B cell-enriched regions in lymphoid tissues. Further studies will focus on understanding the regulation of BL-CAM expression during B cell development and differentiation.

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